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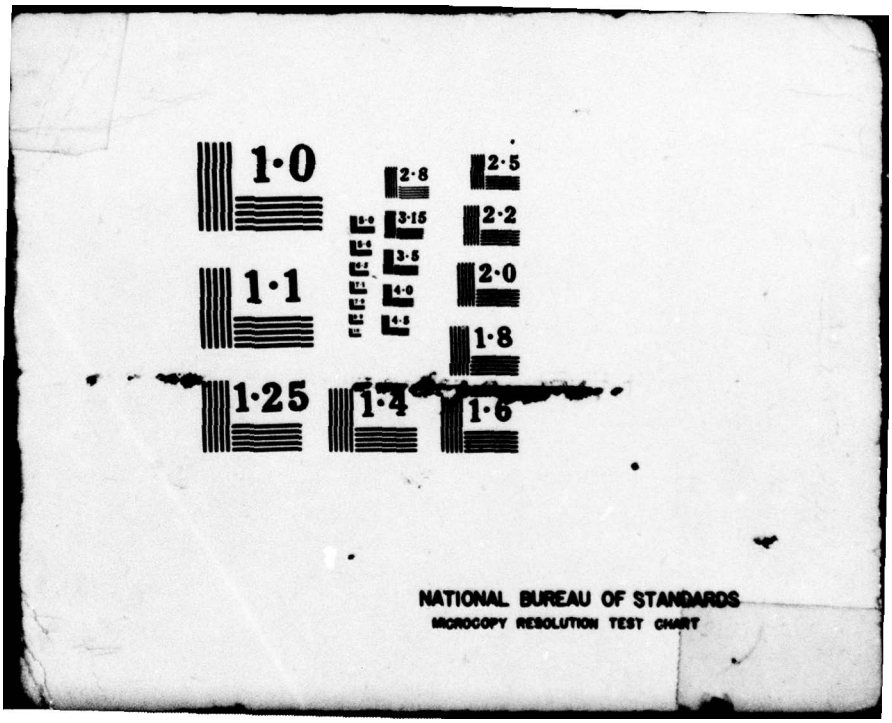
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# Acquired Immunity to Pathogenic Fungi in Gnotobiotic Animals



Annual Progress Report

February 1979

(June 1977 - February 1978)



Edward Balish, Ph.D.

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During primary T. mentagrophytes infection of Strain 2 guinea pigs, the colony forming units (CFU) of fungi in the lesion peaked between days 7 and 14, while the lesion severity peaked between days 11 and 16. Concomitant with the latter peak, a pronounced depression in the in vitro mitogenic activity of spleen (SPC) and lymph node cells (LNC) was observed. Only after resolution of the primary infection (day 21) did LNC show increased DNA synthesis in the presence of fungal antigens. During cutaneous reinfection, there was no

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distinct peak fungal load and CFU appeared to decrease steadily during the accelerated course of a reinfection disease. Lymph node cells from guinea pigs with severe, ulcerated reinfection lesions generally exhibited a heightened response to fungal antigen *in vitro*. The LNC from guinea pigs with mild reinfection dermatophytosis had depressed *in vitro* reactivity to mitogens and dermatophyte antigen. The suppression of blastogenic activity during dermatophyte infection appeared to be associated with autologous serum (AS) components since increased DNA synthesis resulted when SPC or LNC were cultured with fetal calf serum (FCS). The depressed *in vitro* DNA synthesis of lymphocytes (cultured with dermatophyte antigens) that were harvested during reinfection did not correlate with an impaired ability of infected guinea pigs to respond with a delayed-type hypersensitivity (DTH) skin test *in vivo*. These results support the hypothesis that experimental *T. mentagrophytes* dermatophytosis is a cell-mediated hypersensitivity disease that can be modified by immunosuppressive control mechanisms elaborated, or induced by, the fungus.

# Acquired Immunity to Pathogenic Fungi in Gnotobiotic Animals

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By

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## INTRODUCTION

The precise role of cell-mediated immune (CMI) responses in host resistance to dermatophytosis remains poorly understood. Lesion clearance has been shown to be preceded temporally by the onset of delayed-type hypersensivity (DTH) and in vitro blastogenesis of regional lymph node cells to specific fungal antigens (5,9). Cruickshank, Trotter and Wood (3) induced a secondary immune response to fungal infection by sensitization of guinea pigs with acetone-extracted mycelia in complete Freund's adjuvant (CFA) and they also adoptively transferred DTH with peritoneal exudate cells. Recently, Tagami and co-workers (17,18) have shown that Trichophyton-infected guinea pigs and humans manifest contact sensitivity to a trichophytin patch test. Despite the association of these CMI responses to fungal antigen with infection, the extent to which hypersensitivity mechanisms alter the course of infection or disease are unknown.

In the present studies, we attempted to correlate the number of infecting fungi in the lesion site with the in vitro blastogenic responses of spleen (SPC) and lymph node cells (LNC) to polyclonal mitogens and specific dermatophyte antigens at different stages of primary challenge and reinfection of guinea pigs with T. mentagrophytes.

## MATERIALS AND METHODS

Animals and Infection. Randomly-bred, closed colony Strain 2 guinea pigs weighing at least 300 grams were shaved on the back with electric clippers and infected with a 2-3 week old mycelial culture of Trichophyton mentagrophytes var. granulosum (ATCC 18748) by rubbing the organism onto the skin with a cotton swab. At weekly intervals after infection animals were selected for sacrifice, either randomly or based upon skin test response, to obtain skin samples and secondary lymphoid organs.

Fungal Enumeration. Animals were sacrificed by cardiac puncture and exsanguination. Square centimeters of skin (two/animal) were excised from the lesion area and ground in Teflon/glass tissue homogenizers in 5 ml sterile saline. Homogenates were duplicate plated in serial ten-fold dilutions on agar plates modified from Carlisle et al. (2). Briefly, the growth medium consisted of Mycosel agar (BBL, Cockeysville, Md.), 36 g/liter, 15% fetal calf serum (FCS), and .02% bromthymol blue. The pH of the medium was adjusted to 7.6-7.8 with 1N NaOH. Colonies were counted after 5-7 days incubation at room temperature and reported as mean colony forming units (CFU/$cm^2$) of skin.

Trichophytin Antigens. Cultures of T. mentagrophytes were grown at 25°C in 2 liter flasks containing 400-1000 ml of fluid Sabouraud's medium (GIBCO, Grand Island, N.Y.). Cultures were incubated on a shaker for about 90 days prior to preparation of two antigens as follows: (i) an acetone-dried, particulate antigen (PART AG) was prepared by dehydration with three changes of acetone (3,13), and grinding the harvested cells in a mortar and pestal to a fine powder; and (ii) a soluble antigen (SOL AG) was prepared by autoclaving (15 min, 121°C) and centrifuging (~350 x g, 10 min) the culture supernatant. Antigen protein concentrations were estimated by the method of Lowry et al. (11).

In Vitro Blastogenesis Assays. Cell suspensions were made from the spleen or pool of lymph nodes (suprascapular, axillary and cervical-tracheal) from each animal by gentle teasing and passage through a 60-gauge mesh steel screen. Cells were washed twice in phosphate buffered saline (PBS) and resuspended in RPMI 1640 medium (supplemented with penicillin and streptomycin) at a concentration of $2 \times 10^6$/ml with 10% heat-inactivated autologous serum (AS) or FCS. Quadruplicate wells of a microtiter plate (Falcon, Oxnard, Calif.) received 0.1 ml of cells and 0.1 ml of specific antigen or the following mitogens (diluted in RPMI 1640 medium): phytohemagglutinin M (PHA; Difco Laboratories, Detroit, Mich.), 200-400 µg/ml; concanavalin A (ConA; Calbiochem, San Diego, Calif.), 20-100 µg/ml; pokeweed mitogen (PWM; Gibco, Grand Island, N.Y.), 50-100 µg/ml; or lipopolysaccharide W (LPS) from E. coli (Difco) 500-1000 µg/ml. Spleen cells and lymph node cells were cultured for 96 hours in flat-bottomed microtiter plates (Falcon, Oxnard, Calif.) and then pulsed with 1 µCi [$^3$H]thymidine (in 0.05 ml of RPMI 1640 medium) for an additional 18 hours. Cells were harvested by precipitation onto glass fiber filters and the trapped radioactivity was counted in a liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Blastogenic responses were reported as mean $\log_{10}$ for the concentration of antigen or mitogen causing the highest stimulation in counts per minute (cpm) from two or three concentrations of mitogen or antigen used in each assay.

Skin Testing. Guinea pigs were shaved and 32 µg/ml of the soluble trichophytin (SOL AG), in 0.1 ml of pyrogen-free saline (Elkins-Sinn, Cherry Hill, N.J.), was injected intradermally (I.D.) into the flank. The diameter of erythema, in mm, was measured in two directions, and the induration (double skin thickness) was assessed using a Schnelltaster (H.T. Kroplin, Hessen, Germany). Measurements were made at 30 min, 4, 24 and 48 hours, but only the peak 24 hour responses are reported.

Statistical Analysis. Colony forming units and cpm were converted to $\log_{10}$ values prior to calculation of the mean, standard deviation (S.D.) and standard error (S.E.). Significant differences were evaluated by a two-tailed F-test in the analysis of variance (14).

## RESULTS

The Course of the Dermatophyte Lesion. Following infection, animals were observed daily for macroscopic inflammation, tissue damage and healing. Lesion development and evolution closely resembled previous reports of experimental dermatophytosis in the guinea pig (6,8).

Primary lesions were routinely reproducible. Lesion areas first became evident with erythema 4-5 days after initial infection (Table 1). The intensity of the erythema and the lesion size increased until day 9 when the inflamed skin became covered with a flaky scale. Within 24-48 hours the lesions ulcerated, often with serous weeping, indicating peak tissue damage. Within 24 hours after ulceration, the lesions crusted over and began drying out. Erythema surrounding the lesions gradually subsided prior to day 21 and drying crusts sloughed off leaving smooth scars. Healing continued and dense hair grew back rapidly into the scars by 5 weeks.

Animals were reinfected a few weeks or months following spontaneous recovery from the initial infection in an area away from the primary scar (usually in the opposite flank). Previous investigators have reported difficulty in reinfecting the skin area over the primary lesion (4). Although secondary lesions underwent a similar sequence of disease development, inflammation was evident within 24-48 hours, most lesions appeared to peak almost

1 week earlier (day 6-7) and there was less tissue damage (Table 1). Also, some animals with a secondary infection had a very mild erythema which never developed into an ulcerative lesion. The lesion variability between animals was more noticeable during secondary infection than during primary infection.

Fungal Enumeration. In order to quantitate the growth of the fungi in the lesion sites during primary and secondary infection, skin samples were excised, homogenized and plated out to measure viable fungi.

Primary cutaneous T. mentagrophytes infections were initiated at low and high challenge levels and groups of animals were sacrificed at specified intervals for skin cultures (Table 2). At a lower challenge level ($\sim 10^2$), the fungal load per $cm^2$ of skin increased during the first week after infection, while at the higher challenge level ($\sim 10^5$) there was a reduction in the viable count 6 days after challenge. Fungal loads peaked at $10^5$-$10^6$ CFU/$cm^2$ between days 7-11 and declined during the next 2 weeks. Low levels of organisms were isolated from healed lesion scars 4 weeks after infection (Table 2).

Animals were reinfected (cutaneous) approximately 3 weeks after healing of the primary lesion site. The rechallenge infectious dose was about $10^4$ CFU. By day 7, the fungal load had decreased (from day 0) and was about 1 log lower than the fungal load observed 6 days after a high ($10^5$) primary challenge (Table 2). Unlike the primary infections, CFU of T. mentagrophytes continued to decline during the second week of reinfection. By day 14, colony counts from reinfected skin were 1-2 logs lower ($p < 0.01$) than those detected 14 days after a primary infection (Table 2). At day 21 of a reinfection, viable fungi still persisted in skin at levels that corresponded to days 21 and 28 of the primary infections (Table 2).

In Vitro Blastogenic Response During Primary Infection. In order to assess the induction of acquired-immunity, SPC and LNC were isolated and cultured in vitro with two specific T. mentagrophytes antigens on days 0, 7, 14, and 21 following a cutaneous infection. Polyclonal T- and B-cell mitogens were included as controls to monitor the ability of SPC and LNC to respond to nonspecific stimulation.

Day 0 stimulations (mean $\log_{10}$ cpm) were those of lymphocytes from guinea pigs that were shaved and infected shortly before sacrifice (Table 3). Only minor changes (from the day 0 blastogenesis) were seen in the mitogenic responses SPC and LNC after 1 week of infection (day 7, Table 3).

The most striking result obtained by testing the blastogenic response of SPC and LNC to polyclonal mitogens during the primary infection was a significant depression ($p < .01$) of blastogenesis on day 14 of the infection at the peak of fungal load and tissue damage. This depression was reflected in the response of both SPC and LNC and was at least a 1 log decrease in cpms for the four T- or B-cell mitogens tested (Table 3). As the lesions healed, all mitogenic responses increased toward day 0 values (Table 3).

On days 0, 7 and 14 neither SPC nor LNC manifested a blastogenic response to dermatophyte antigens. The only positive blastogenic responses to dermatophyte antigens occurred 3 weeks after initiation of the primary infection and only LNC cells responded to both SOL AG and PART AG. There was no significant in vitro proliferative response by SPC to trichophytin antigen on any day tested during a primary infection (Table 3).

In Vitro Blastogenic Response During Secondary Infection. We also measured the DNA synthesis of SPC and LNC during reinfection in two groups of animals that had recovered from a primary T. mentagrophytes infection. In the first experiment, a group of animals was cutaneously infected 6 months

after clearance of the primary infection and sacrificed for *in vitro* blastogenesis on days 0, 7, 14, and 21 (Table 4) after challenge.

On day 0, the SPC and LNC responded within previously observed normal ranges to polyclonal T- and B-cell mitogens. In addition, as observed on day 21 of the primary infection, only LNC were capable of responding significantly to both trichophytin antigens (Table 4).

By the end of the first week, it was evident from the lesions that there was a wide range of biological variability in the animals' response to reinfection. Some animals had mild, but erythematous responses (6/17), while others had severe bleeding ulcerations (11/17). In order to determine whether these different macroscopic lesions correlated with a difference in *in vitro* blastogenic response, a single animal from each group (i.e., erythema vs ulceration) was sacrificed on day 7 after reinfection (Table 4). Spleen cell blastogenic responses to both mitogens and antigens were similar in both animals; however, the LNC blastogenic responses to fungal antigens differed significantly. Lymph nodes from the animal with the severe ulcerated lesion responded positively to the fungal antigens, while the animal with a mild skin lesion had no significant blastogenic response to the same antigen (Table 4). Thus, cutaneous ulceration seemed to correlate with a heightened blastogenic response of LNC during reinfection. At this point, we suspected that the animals with mild lesions were undergoing a primary instead of secondary infection, but they never developed the ulcerated lesions that were typical of primary dermatophyte infections.

Animals sacrificed on day 14 after reinfection had especially severe skin lesions and they also had LNC blastogenic responses to fungal antigens that exceeded day 0 values by over 1 log; however, no dramatic increase was evident in SPC response to the same antigens (Table 4).

Animals sacrificed on day 21 after reinfection were in the healing phase and exhibited dry scars in skin lesion areas. The SPC and LNC of the latter animals showed no significant DNA synthesis in the presence of fungal antigen (Table 4).

In an attempt to control the large degree of variability observed in the previous experiment, another group of 12 guinea pigs, which had recently recovered from a primary infection, was skin tested with soluble trichophytin antigen (Table 5, Exp. 1). One week after skin testing, all animals were cutaneously reinfected with *T. mentagrophytes* and grouped for sacrifice on the basis of the magnitude of their delayed (24 hours) skin test (DTH) reactivity (Table 6).

The very low DTH responders were sacrificed on day 0 and they manifested mitogenic responses that were within previously observed normal ranges (Table 7, Group 1). As in the previous reinfection study (Table 4), only LNC responded to both fungal antigens.

On reinfection day 7, the next higher group (2a, Table 7) of DTH responders was sacrificed. The gross lesions in this group of low DTH responders were mild on day 7. A marked depression in the ability of the latter animals' LNC to proliferate in the presence of polyclonal T- and B-cell mitogens was observed (Table 7, Group 2a). Also, SPC from the latter group manifested a depressed mitogenic response to ConA and PHA.

The third group of guinea pigs, sacrificed 14 days after challenge and demonstrating moderate lesions (Table 7, Group 3a), had SPC blastogenic responses (to mitogens and fungal antigens) that were essentially similar to day 0 values. The mitogen-induced blastogenesis of LNC from group 3a animals (Table 7) were also similar to day 0 values, but the LNC blastogenic

responses to fungal antigens were decreased to insignificant levels in the presence of autologous serum (AS). The inhibition of blastogensis by AS suggested that an inhibitory factor might be present in the serum of dermatophyte infected guinea pig.

The highest DTH responders (Table 7, Group 4a) elicited the largest and most severe secondary lesions. In spite of early lesion severity, rapid healing (indicative of an accelerated secondary infection) commenced much earlier (day 11). The mitogenic response from these animals' LNC (Group 4a) was nonspecifically depressed when compared to day 0 control values and neither soluble nor particulate fungal antigen evoked significant incorporation of [$^3$H]thymidine in the presence of AS. Thus, there was no clear correlation between in vivo DTH skin test reactivity prior to reinfection and positive in vitro blastogenesis to fungal antigens during reinfection.

Evidence That Autologous Serum (AS) Is Able To Depress Lymphocyte Responsiveness In Vitro. In the previous experiment, SPC and LNC were cocultured in the presence of 5% FCS or AS on days 7, 14, and 21 following reinfection in order to compare directly the ability of guinea pig lymphocytes to respond in a culture medium with either heterologous or autologous serum supplementation (Table 7).

On reinfection day 7 (Group 2b), incorporation of [$^3$H]thymidine was significantly increased in unstimulated, mitogenically stimulated and specific antigen stimulated cultures in the presence of FCS. Whereas, the LNC responses to all mitogens were significantly increased in FCS; only PWM and LPS blastogenesis of SPC (Group 2b) were enhanced by FCS. Dermatophyte antigens (SOL and PART) were not significantly different from unstimulated controls, indicating a loss of antigen specific activity during the first week of reinfection.

On reinfection day 14 (Table 7, Group 3b) and 21 (Table 7, Group 4b), FCS did not increase the baseline incorporation of [$^3$H]thymidine with unstimulated SPC or LNC. However, the LNC responsiveness to both soluble and particulate T. mentagrophytes antigen was "uncovered" ($p < .01$) by culturing in FCS instead of AS supplemented medium (Group 3a and 4a). Certain mitogenic responses of LNC were increased in the presence of FCS (PWM and LPS), but others were depressed (day 14, ConA). When cultured in FCS supplemented medium, spleen cell responses (on day 14) were generally less enhanced than LNC.

Evidence That DTH Skin Test Does Not Correlate With In Vitro Depression of Lymphocyte Reactivity. Since the capacity of LNC to respond to in vitro stimulation with specific T. mentagrophytes antigens was reduced during the first week of reinfection (Table 7), the following experiment was designed to determine if the DTH skin test response in vivo was similarly depressed at day 7 following reinfection.

Another group of guinea pigs was cutaneously infected with T. mentagrophytes and skin tested about 1 week following clearance of the primary lesions (Table 5, Exp. 2). One week after skin testing, all animals were shaved and cutaneously reinfected. On day 7 of the secondary infection, the animals were skin tested again on the flank, posterior to the secondary lesion (Table 5, Exp. 3). There was no significant change in the ability of these animals to respond with DTH to intradermal injection of soluble T. mentagrophytes while undergoing a secondary infection (Table 7).

## DISCUSSION

The self-limiting course of T. mentagrophytes infection in inexperienced guinea pigs has been reported previously (3,6,9). We observed that primary lesions started with early inflammation (day 5 after infection) that developed into open bleeding ulcerations at the peak of infection (days 11-16). This rapid inflammation and lesion severity was most likely accounted for by our repeated in vivo passage of the organism, and our use of mycelial inocula rather than spores. The genesis of symptomatology in experimental dermatophytosis has been suggested to be due to active mycelial invasion of the area above the stratum granulosum (7), and a mycelial inoculum would be expected to initiate disease more rapidly and result in higher fungal load prior to host sensitization by eliminating the time and conditions required for the germination of spores.

Although other investigators have quantitated the spore inoculum used to initiate infection (8), the subsequent fungal growth during primary infection has been evaluated only by microscopic examination or by qualitative culturing of skin scrapings (4,5,8). Our results indicate that it is possible to quantitatively follow fungal proliferation and elimination during lesion development by homogenizing and culturing infected skin. The main limitation of the homogenization technique is that the morphology of the cells forming each CFU is not known. For example, arthrospore or microaleuriospore formation at the peak of infection might result in an increased number of colonies (CFU).

The time course of the developing primary lesions closely paralleled increasing cutaneous fungal growth (CFU). Lesion severity, in terms of gross tissue damage, occurred at the same time as the peak fungal load (day 14). Shortly thereafter, lesion erythema and ulceration decreased and healing began. Viable skin fungi (CFU) also declined between the second and third week of primary infection. Although fungi were still cultured from the skin 3-4 weeks after initiation of infection, lesions were healing and there was no noticeable erythema. These observations suggest that either the fungi are inactivated following serous ulceration, possibly by serum inhibitory factors (2), and thus prevented from further producing inflammatory fungal products, or that the host's immunoregulatory system depresses the immune response to the persistent fungal antigens.

The correlation between lesion development and fungal enumeration during reinfection is less clear. Fungal colonies decreased steadily during the first 3 weeks of reinfection, while macroscopic lesions peaked in severity after 1 week. The host's response to the reinfection, characterized by rapid development of erythema, was not capable of precluding severe ulcerations similar to those observed during primary infection. Thus, reinfection of guinea pigs resulted in an accelerated lesion development apparently because the presensitization period, observed during primary infection, was not present. The fact that no clearcut peak fungal load corresponded with peak tissue damage (days 5-7) during reinfection suggests that the severity of the secondary lesion may be the result of an anamnestic hypersensitivity reaction.

There was only a single attempt reported to culture lesions during a secondary T. mentagrophytes infection of guinea pigs (4), and the organisms were detected only by direct microscopic examination. Fungi were observed only out to day 8. Our results suggest that skin homogenization may be a more sensitive method of determining fungal persistence, since organisms and

spores have been reported to persist in deep hair follicles (10). It is interesting to note that although secondary lesions developed more rapidly and peaked about 1 week earlier than after primary inoculation, there were still culturable fungi, in skin, on days 14 and 21 after reinfection.

Specific in vitro lymphocyte transformation to trichophytin was demonstrated in LNC cultures during primary and secondary infection. During primary infection, a positive response was not detected until day 21. These results differed from those of Kerbs, Greenberg and Jesrani (9) who were able to demonstrate in vitro responses to T. mentagrophytes antigen on days 9, 11, 13, and 15 when cultured with normal guinea pig serum.

Spleen cells did not respond to fungal antigens at any time point tested. Other investigators have interpreted a similar lack of spleen cell proliferation to antigen in vitro as evidence for a population of suppressor cells (12). In light of the depression of antigen specific blastogenesis during reinfection (Table 4, day 21; Table 7, days 7, 14, and 21), it is possible that some population of cells is producing a suppressor factor or factors. This does not exclude the possibility that suppressor cells may also be present in lymph nodes, just that those in the spleen predominate in vitro.

During reinfection, the only classical secondary response to fungal antigen, as detected by blastogenesis of LNC in vitro, was on days 7 and 14 following a 6 month interval after the primary infection. The secondary lesions that developed on these animals were severely ulcerated. Rather than correlating with any enhanced immunity, these increased in vitro LNC proliferations were indicative of an exaggerated hypersensitivity response even though fungal colonies were decreasing over the 3 week course of infection. In a second reinfection experiment, none of the animals had a blastogenic response to fungal antigen significantly greater than the residual sensitivity remaining from the more recent primary infection suggesting the possibility that T. mentagrophytes-induced immunoregulatory mechanisms may deteriorate over time. The apparent absence of a classical secondary response, as measured by DNA synthesis in regional lymph nodes, has been described previously during challenge with contact agents (1).

The decreased response of both SPC and LNC to polyclonal T- and B-cell mitogens during infection and reinfection, indicate a pronounced nonspecific depression of reactivity. During the primary infection, this depression was most evident on day 14 which correlated well with the peak fungal load and lesion ulceration. During reinfection, it occurred 1 week earlier (day 7) which also correlated timewise with peak inflammation, but not peak fungal load.

Various hypotheses have been proposed to explain the activation and expression of immune suppression. Asherson and Zembala (1) have postulated a feedback mechanism in which suppressor cells respond to an antigen overload interacting with sensitized lymphocytes. Turk, Polak and Parker (19) have proposed that immunoregulation is designed to balance effector function in order to preclude systemic tissue damage.

Data presented on culturing both SPC and LNC with FCS provide strong evidence that both antigen specific and nonspecific suppressor factors are present in the AS during reinfection. It would be reasonable to assume that the differences observed in vitro with our LNC cultures from those by Kerbs et al. (9) during primary infection might be due to their reported use of normal guinea pig serum. The use of FCS resulted in increased responses to polyclonal mitogens and also uncovered significant DNA synthesis of LNC to both fungal antigens on days 14 and 21 of reinfection. Walters et al. (30)

reported the presence of serum factors which specifically blocked lymphocyte reactivity *in vitro* in patients chronically infected with dermatophytosis. Our experiments support a hypothesis that a host-immune response is required to resolve these infections; an exaggerated or premature production of serum inhibitory factors might be able to interfere with this apparent acquired-immunity.

Generally, suppression of cell-mediated immunity during fungal infections is detected by a lack of skin test reactivity or anergy associated with chronic or progressive infection (15). Our data indicated that the depressed *in vitro* responses measured on day 7 of reinfection do not correlate with a similar depression of intradermal DTH. Thus, the intradermal skin test response alone did not fully reflect the host's capacity to respond to fungal antigens during active infection.

The results of the experiments presented in this paper support the hypothesis of Sulzberger (16) that dermatophytosis is primarily a hypersensitivity disease which results in local tissue damage. This hypersensitivity may also be integrally required for inactivation of the fungi and resolution of the disease. The early erythema observed during primary infection (days 5-9), prior to detectable systemic sensitization of the host, raises the possibility of a primary irritant or toxic effect of the fungal antigens which later result in sensitization of the host. The rapid inflammation, which developed upon cutaneous reinfection was compatible with biological contact sensitivity. It was difficult to assess, however, precisely when this contact-like sensitivity first influenced the primary lesion.

Our results, also, indicated that pronounced nonspecific and specific suppression of lymphocyte transformation occurred during the course of primary and secondary infection with *T. mentagrophytes* and this suppression was related to inhibitory serum factors. How this observed suppression *in vitro* relates to the resolution of the infection and regulation of host-immunity *in vivo* requires further investigation.

## LITERATURE CITED


1. Asherson, G.L., and M. Zembala. 1976. Suppressor T-cells in cell-mediated immunity. Br. Med. Bull. 32:158-164.

2. Carlisle, D.H., J.C. Inouye, R.D. King, and H.E. Jones. 1974. Significance of serum fungal inhibitory factor in dermatophytosis. J. Invest. Dermatol. 63:239-241.

3. Cruickshank, D.N.D., M.D. Trotter, and S.R. Wood. 1960. Studies on trichophytin sensitivity. J. Invest. Dermatol. 35:219-223.

4. Delamater, E.D. 1941. Experimental studies with the dermatophytes. III. Development and duration of hypersensitivity in guinea pigs. J. Invest. Dermatol. 4:143-158.

5. Delamater, E.D., and R.W. Benham. 1938. Experimental studies with the dermatophytes. II. Immunity and hypersensitivity produced in laboratory animals. J. Invest. Dermatol. 1:469-488.

6. Delamater, E.D., and R.W. Benham. 1938. Experimental studies with the dermatophytes. I. Primary disease in laboratory animals. J. Invest. Dermatol. 1:451-467.

7. Desai, S.C., and M.L.A. Bhat. 1960. Studies on experimental infections with T. rubrum in humans and the mechanism of griseofulvin effect. J. Invest. Dermatol. 35:297-303.

8. Greenberg, J.H., R.D. King, S. Kerbs, and R. Field. 1976. A quantitative dermatophyte infection model in the guinea pig--a parallel to the quantitated human infection model. J. Invest. Dermatol. 67:704-708.

9. Kerbs, S., J. Greenberg, and K. Jesrani. 1977. Temporal correlation of lymphocyte blastogenesis, skin test responses and erythema during dermatophyte infections. Clin. Exp. Immun. 27:526-530.

10. Kligman, A.M. 1955. Tinea capitis due to M. audouinii and M. canis. Arch. Derm. 71:313-337.

11. Lowry, D.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1952. Protein measurement with the Folen-Phenol reagent. J. Biol. Chem. 193:265-275.

12. Neta, R., and S.B. Salvin. 1976. T and B lymphocytes in the regulation of delayed hypersensitivity. J. Immunol. 117:2014-2020.

13. Ottavano, P., H. Jones, J. Jaeger, R. King, and D. Bibel. 1974. Trichophytin extraction: Biological comparison of trichophytin extracted from Trichophyton mentagrophytes grown in complex medium and a defined medium. Appl. Microbiol. 28:271-276.

14. Steel, R.G.D., and J.H. Torrie. 1970. Analysis of Variance, pp. 99-131. In: Principles and Procedures of Statistics, McGraw-Hill Book Co., Inc., N.Y.

15. Stobo, J.D., and C.P. Loehnen. 1966. Immunoregulation and autoimmunity. Mayo Clin. Proc. 51:479-483.

16. Sulzberger, M.B. 1950. Immunologic changes brought about by fungi and fungus products. Ann. N.Y. Acad. Sci. 50:767-772.

17. Tagami, H., S. Watanabe, S. Ofuji, and K. Minami. 1977. Trichophytin contact sensitivity in patients with dermatophytosis. Arch. Dermatol. 113:1409-1414.

18. Tagami, H., S. Watanabe, and S. Ofuji. 1973. Trichophytin contact sensitivity in guinea prep with experimental dermatophytosis induced by a new inoculation method. J. Invest. Dermatol. 61:237-241.

19. Turk, J.L., L. Polak, and D. Parker. 1976. Control Mechanisms in delayed-type hypersensitivity. Brit. Med. Bull. 32:165-170.

20. Walters, B.A.J., J.E.D. Chick, and W.J. Halliday. 1974. Cell-mediated immunity and serum blocking factors in patients with chronic dermatophyte infections. Int. Arch. Allergy. 46:849-857.

Table 1. Stages in the development and resolution of gross lesions during primary and secondary cutaneous T. mentagrophytes infection in guinea pigs

| | Primary | Secondary |
|---|---|---|
| ERYTHEMA/SPREADING | 5-8[a] | 1-7 |
| SCALING (24-48 Hours) | 9-12 | 4-7 |
| SEROUS ULCERATION (24 Hours) | 11-16 | 5-7 |
| CRUST/SCABS | 12-19 | 6-11 |
| SCAR/ALLOPECIA | 18-25 | 10-19 |

[a]Days postinoculation.

Table 2. Viable colony forming units/$cm^2$ skin (CFU) of T. mentagrophytes during primary or secondary cutaneous infection

| | Day 0 | Day 6 | Day 7 | Day 11 | Day 14 | Day 17 | Day 21 | Day 28 |
|---|---|---|---|---|---|---|---|---|
| Primary (low) | 2.00(.22)[a] | N.D.[b] | 5.19(.23) | N.D. | 4.45(.27) | N.D. | 2.32(.31) | N.D. |
| Primary (high) | 4.98(.04) | 4.19(.25) | N.D. | 6.25(.13) | N.D. | 4.90(.30) | N.D. | 1.72(.64) |
| Secondary | 4.22(.22) | N.D. | 3.11(.31) | N.D. | 2.39(.49) | N.D. | 1.87(.75) | N.D. |

[a]Mean $\log_{10}$ CFU (S.E.).

[b]Not determined.

Table 3. Effect of a primary T. mentagrophytes infection on the blastogenic response of guinea pig spleen (SPC) or lymph node cells (LNC)

| Mitogen or Antigen | Response of SPC After Primary Infection | | | |
|---|---|---|---|---|
| | Day 0 (6)[a] | Day 7 (6) | Day 14 (6) | Day 21 (6) |
| Unstimulated | 2.78 (.11)[b] | 3.06 (.10) | 2.64 (.35) | 3.23 (.15) |
| PHA | 4.58 (.31) | 4.44 (.53) | 3.35 (.63)[d] | 4.68 (.26) |
| CON A | 5.02 (.25) | 4.63 (.62) | 3.29 (.50)[d] | 4.84 (.25) |
| PWM | 3.93 (.15) | 4.26 (.68) | 2.97 (.51)[d] | 3.97 (.58) |
| LPS | 3.22 (.42) | 3.49 (.42) | 2.74 (.27)[d] | 3.29 (.29) |
| SOL AG | 2.94 (.30) | 3.24 (.45) | 2.84 (.38) | 3.24 (.25) |
| PART AG | 2.85 (.44) | 3.41 (.43) | 2.94 (.40) | 3.20 (.30) |
| Mitogen or Antigen | Response of LNC After Primary Infection | | | |
| | Day 0 (3) | Day 7 (3) | Day 14 (3) | Day 21 (3) |
| Unstimulated | 2.35 (.21) | 2.34 (.14) | 2.17 (.17) | 2.34 (.15) |
| PHA | 4.91 (.13) | 4.66 (.56) | 2.85 (.70)[d] | 4.79 (.09) |
| CON A | 4.87 (.30) | 4.91 (.19) | 2.91 (1.09)[d] | 4.48 (.40) |
| PWM | 4.67 (.59) | 4.35 (.66) | 2.41 (1.02)[d] | 4.68 (.13) |
| LPS | 3.05 (.16) | 2.90 (.39) | 2.26 (.30)[d] | 2.94 (.44) |
| SOL AG | 2.82 (.37) | 2.55 (.22) | 2.40 (.10) | 3.07 (.40)[c] |
| PART AG | 2.53 (.36) | 2.44 (.13) | 2.23 (.44) | 3.47 (.34)[c] |

[a]Number of animals tested.

[b]Mean $\log_{10}$ cpm (Standard Deviation).

[c]Values differ significantly from unstimulated value: ($p < .01$).

[d]Values depressed significantly with respect to days 0, 7 and 21 ($p < .01$).

Table 4. Effect of a secondary T. mentagrophytes infection on the blastogenic response of guinea pigs spleen (SPC) and lymph node cells (LNC). Infection was initiated about six months following clearance of a primary infection

| Mitogen or Antigen | SPC Response After Secondary Infection | | | |
|---|---|---|---|---|
| | Day 0 (3)[a] | Day 7 (2) | Day 14 (3) | Day 21 (2) |
| Unstimulated | 3.27 (.46)[b] | 2.80/2.77[d] | 3.23 (.08) | 3.46/3.09[d] |
| PHA | 4.64 (.22) | 4.25/3.67 | 4.35 (.22) | 4.76/4.39 |
| CON A | 4.69 (.41) | 4.37/3.83 | 4.21 (.21) | 5.01/4.49 |
| PWM | 3.70 (.41) | 3.36/3.81 | 4.62 (.32) | 4.25/4.02 |
| LPS | 3.46 (.56) | 2.47/2.64 | 3.34 (.36) | 3.49/3.71 |
| SOL AG | 3.23 (.45) | 2.90/2.99 | 3.33 (.20) | 3.44/3.06 |
| PART AG | 3.36 (.34) | 2.86/2.94 | 3.54 (.38) | 3.25/2.84 |
| Mitogen or Antigen | LNC Response After Secondary Infection | | | |
| | Day 0 (3) | Day 7 (2) | Day 14 (3) | Day 21 (2) |
| Unstimulated | 2.62 (.01) | 2.89/2.42 | 2.83 (.20) | 2.30/2.37 |
| PHA | 4.67 (.11) | 4.40/3.81 | 4.32 (.71) | 4.86/4.20 |
| CON A | 4.13 (1.29) | 4.33/3.74 | 4.09 (.14) | 4.51/2.84 |
| PWM | 3.35 (.76) | 4.95/4.08 | 4.94 (.16) | 3.96/3.32 |
| LPS | 2.75 (.28) | 3.70/2.38 | 3.43 (.15) | 3.54/3.56 |
| SOL AG | 2.92 (.19)[c] | 4.44[c]/2.60 | 3.49 (.21)[c] | 2.85/2.86 |
| PART AG | 3.16 (.63)[c] | 4.71[c]/2.69 | 4.27 (.29)[c] | 3.16/2.67 |

[a]Number of animals tested.

[b]Mean $\log_{10}$ cpm incorporated (Standard Deviation).

[c]Significant increase over unstimulated control cells ($p > .01$).

[d]Each animal reported individually (mean $\log_{10}$ cpm).

Table 5. Delayed-type hypersensitivity (24 hr) skin test response to soluble *T. mentagrophytes* antigen (32 µg/0.1 ml) following primary or secondary infection

| Exp. No. | Weeks Following Infection | No. of Animals | Erythema | Induration |
|---|---|---|---|---|
| 1 | 7-10 weeks/primary | 12 | 11.71 (1.12)[a]<br>(8-19)[b] | .92 (.20)[a]<br>(.3-1.7)[b] |
| 2 | 8 weeks/primary | 10 | 11.00 (.56)<br>(9-15) | .69 (.10)<br>(.3-1.2) |
| 3 | 1 week/secondary | 8 | 12.69 (.55)<br>(11-15) | .68 (.22)<br>(.1-1.8) |

[a]Mean and Standard Error (mm).

[b]Range (mm).

Table 6. Cutaneous DTH (24 hr) skin test reactivity to soluble trichophytin antigen one week prior to reinfection.

| Skin test Response | (1)[b] Day 0 | (2) Day 7 | (3) Day 14 | (4) Day 21 |
|---|---|---|---|---|
| Erythema (mm) | 8.33 (.70)[a] | 11.0 (.82) | 13.17 (.68) | 16.0 (1.42) |
| Induration (mm) | .40 (.06)[a] | .50 (.15) | 1.00 (.38) | 1.77 (.35) |

[a]Mean and Standard Error of three guinea pigs per group.

[b]Sacrifice groups correspond to animals in Table 7.

Table 7. Effect of a secondary *T. mentagrophytes* infection on the blastogenic response of guinea pig spleens (SPC) and lymph node cells (LNC) cultured in heat inactivated autologous serum (AS) or fetal calf serum (FCS)

| Mitogen or Antigen | Response of Spleen Cells After Infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2a | 2b | 3a | 3b | 4a | 4b |
| | Day 0 (AS)[a] | Day 7 (AS) | Day 7 (FCS)[b] | Day 14 (AS) | Day 14 (FCS) | Day 21 (AS) | Day 21 (FCS) |
| Unstimulated | 2.62 (.36) | 2.49 (.13) | 2.79 (.12)[d] | 2.91 (.17) | 2.59 (.04) | 2.74 (.33) | 2.60 (.22) |
| PHA | 3.87 (.55) | 3.73 (.28) | 3.37 (.25) | 3.61 (.24) | 3.69 (.37) | 3.29 (.67) | 4.13 (.23)[d] |
| CON A | 4.36 (.29) | 3.13 (.58) | 2.83 (.33) | 4.09 (.25) | 2.63 (.12)[f] | 3.29 (1.52) | 2.68 (.07) |
| PWM | 3.70 (.40) | 2.92 (.16) | 3.98 (.28)[d] | 3.39 (.17) | 3.43 (.21) | 3.00 (.49) | 3.34 (.17) |
| LPS | 2.85 (.12) | 2.89 (.21) | 3.13 (.60)[d] | 2.91 (.08) | 3.13 (.05)[d] | 2.63 (.11) | 2.75 (.17) |
| SOL AG | 2.65 (.04) | 2.27 (.47) | 3.11 (.54)[d] | 2.54 (.24) | 2.57 (.07) | 2.51 (.36) | 2.67 (.19) |
| PART AG | 2.75 (.06) | 2.41 (.65) | 2.80 (.57)[d] | 2.64 (.22) | 2.58 (.02) | 2.53 (.28) | 2.70 (.21) |
| | Response of Lymph Node Cells After Infection | | | | | | |
| | Day 0 (AS) | Day 7 (AS) | Day 7 (FCS) | Day 14 (AS) | Day 14 (FCS) | Day 21 (AS) | Day 21 (FCS) |
| Unstimulated | 2.42 (.11) | 2.17 (.08) | 2.85 (.39)[d] | 2.16 (.22) | 2.37 (.14) | 2.49 (.44) | 1.91 (.03) |
| PHA | 4.53 (.40) | 2.79 (.45) | 4.27 (.28)[d] | 4.73 (.07) | 4.71 (.10) | 3.02 (.59) | 4.52 (.36)[d] |
| CON A | 4.18 (.96) | 2.15 (.33) | 2.49 (.41)[d] | 4.40 (.72) | 2.46 (.42)[f] | 3.10 (.76) | 3.29 (1.13) |
| PWM | 4.68 (.28) | 2.23 (.35) | 4.30 (.37)[d] | 3.54 (1.03) | 4.57 (.17)[d] | 2.29 (.03) | 3.67 (.33)[d] |
| LPS | 3.11 (.22) | 2.30 (.36) | 3.67 (.50)[d] | 2.71 (.33) | 3.56 (.04)[d] | 2.54 (.21) | 3.27 (.68)[d] |
| SOL AG | 3.45 (.04)[c] | 2.62 (.98) | 3.11 (.54)[e] | 2.41 (.26) | 3.12 (.02)[cd] | 2.21 (.27) | 2.71 (.64)[cd] |
| PART AG | 3.82 (.32)[c] | 2.17 (.08) | 2.80 (.57)[d] | 2.24 (.31) | 2.93 (.08)[cd] | 2.42 (.05) | 2.91 (.75)[cd] |

[a]Heat-inactivated autologous serum (5%).

[b]Fetal calf serum (5%).

[c]Significant increase over unstimulated ($p < .01$).

[d]Significant increase over autologous serum ($p < .01$).

[e]2/3 Animals significant increase over autologous serum ($p < .01$).

[f]Significant decrease over autologous serum ($p < .01$).

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